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Association of DNA With the Nuclear Lamina in Ehrlich Ascites Tumor Cells

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We have studied in vitro binding of DNA to nuclear lamina structures isolated from Ehrlich ascites tumor cells. At low ionic strength in the presence of Mg^{++}, they bind considerable amounts of mouse and bacterial DNA, forming complexes stable in 2 M NaCl. Single-stranded DNA and pulse-labeled DNA show higher binding efficiencies than native uniformly labeled DNA. When mixing occurs in 2 M NaCl, complex formation is inhibited.

When nuclei are digested with DNAse I under conditions that favor chromatin condensation, DNA associated with matrices subsequently prepared from such nuclei is markedly enriched in satellite DNA. If digestion is carried out with DNAse II while nuclei are decondensed in EDTA, no enrichment in satellite DNA is observed.

Preparations of purified, high-molecular weight, double-stranded DNA contain variable amounts of fast-sedimenting aggregates, which are insoluble in 2 M NaCl but are dispersed by DNA fragmentation or denaturation.

These results point at some artifacts inherent in studies of DNA bound to residual nuclear structures in vivo and suggest conditions expected to avoid these artifacts.

Further, using controlled digestion with DNAse II, we have studied the in vivo association of DNA with nuclear lamina isolated from Ehrlich ascites tumor cells. In the course of DNA fragmentation from above 50 kbp to about 20 kbp average size, the following events were observed. The DNA of high molecular weight (much longer than 50 kbp) behaved as if tightly bound to the nuclear lamina, as judged by sedimentation in sucrose and metrizamide density gradients, electron microscopy, and retention on glass fiber filters. As the size of DNA decreased, it was progressively detached from the nuclear lamina, and at about 20 kbp average length practically all DNA was released. The last 1–4% of DNA, although cosedimenting with the nuclear lamina in sucrose gradients, behaved as free DNA, banding at 1.14 g/cm^3 in metrizamide density gradients and showing less than 4% retention on filters.

At no stage of digestion did the DNA cosedimenting with nuclear lamina show changes in satellite DNA content relative to that of total DNA or enrichment in newly replicated DNA.

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It was shown, however, that digestion of nuclear lamina-DNA complex with EcoRI or Hae III led to the formation of DNA-protein aggregates, which banded at 1.35 g/cm^3 in high salt containing metrizamide density gradients and which were strongly enriched in satellite DNA.

These results argue against the existence of direct tight bonds between DNA and nuclear lamina in vivo but demonstrate that such bonds can be generated under certain conditions in vitro.

Key words: DNA-lamina association, DNA fragmentation, metrizamide gradients, DNA-protein binding in vitro, satellite DNA, chromatin condensation, artifacts

In a number of studies part of the chromosomal DNA of many types of eucaryotic cells has been found to cosediment with residual nuclear structures (RS) derived from interphase nuclei after controlled digestion with DNAse I [1–4], micrococcal nuclease [5–8], restriction enzymes [9–13] or endogenous nuclease [7,14], and extraction of histones in concentrated solutions of NaCl (for review see [15,16]). These findings together with electron microscopic observations have led to the concept that chomatin in the interphase nucleus is attached to non-chromatin proteinaceous skeletal structures and, in this way, is organized in a large number of loops, representing topologically independent domains with possible functional significance (for review see [15,16]). The nature of the attachment sites is not known, but it is believed that DNA and the skeletal proteins form tight hydrophobic bonds, whose stability does not depend on the ionic composition of the medium. Both nuclear lamina (NL) [17] and intranuclear matrix [18,19] have been implicated as chromatin-organizing structures. In many studies, RS-bound DNA has been found enriched in short-pulse labeled DNA [1–3,10,12,14], transcribed DNA sequences [4,13,20], or satellite DNA [8,9,21,22]. However, other studies have argued against the specific association of active genes [11,23] or highly reiterated sequences [7,13,20] with RS.

A question essential for the interpretation of these data is whether the DNA fragments cosedimenting with RS had been really attached to nuclear skeletal elements in vivo. Control experiments designed to check artifactual binding of DNA to RS have given mostly negative results [1,3,14,24,25]. On the other hand, in vitro reconstitution studies have shown that RS proteins are capable of binding DNA [26,27]. The problem has not been studied systematically.

In this work we have investigated some factors which could affect the amount and composition of DNA cosedimenting with RS, namely in vitro binding of DNA to NL, effect of chromatin condensation on the content of satellite DNA in the RS fraction, and occurrence of fast-sedimenting aggregates in solutions of purified DNA. The results of these experiments have allowed us to define the conditions that would avoid some artifacts in this type of study.

Further, taking into account these results, we have studied the association of chromosomal DNA in Ehrlich ascites tumor (EAT) cells with the purified NL fraction, isolated from EAT cells by a method described in [28].

MATERIALS AND METHODS

EAT cells were propagated in mice and labeled with [^{3}H] thymidine for 2 hr (long-term labeling) or shorter periods as described in [28]. *E coli* cells were labeled with [^{3}H] thymine for 24 hr during the logarithmic phase of growth.

Isolation of Nuclear Fraction

EAT cells were washed twice with cold 0.14 M NaCl and suspended in TKM buffer (10 mM Tris·Cl, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.6), containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Nonidet NP-40 was added to 0.5%, and the sample was homogenized with 10 strokes in a glass–Teflon homogenizer. After centrifugation at 1,500g for 10 min, the sediment was washed with TKM buffer and layered on a cushion of 1 M sucrose in TKM buffer. Centrifugation for 10 min at 1,500g yielded a crude nuclear pellet, which was used without further purification.

Isolation of NL Fraction for In Vitro Binding Experiments

This was done using the procedure described in [28] except that the chromatin was digested with 1 unit of DNAse II/A_{260} and 20 μg/ml RNAse for 60 min at 25°C, and the sample was centrifuged through a cushion of 10% sucrose in 2 M NaCl, 10 mM Tris·Cl, 1 mM EDTA, pH 8.0 (HSE), for 30 min at 3,500g. The pellet was dialyzed against 10 mM Tris·Cl, 1 mM EDTA, pH 7.5 (TE), and kept at 4°C after addition of 2 mercaptoethanol to 10 mM and bovine serum albumin (BSA) to 50 μg/ml.

Isolation and Digestion of DNA

The DNA was isolated by the conventional method involving digestion with proteinase K (Merck, 50 μg/ml, 37°C, 18 hr) in the presence of 1% sodium dodecyl sulfate (SDS), 50 mM Tris·Cl, 10 mM NaCl, 1 mM EDTA, pH 8.0, followed by repeated deproteinization with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitation with ethanol. The DNA was dissolved in TE and treated with pancreatic RNAse (10 μg/ml, 2 hr at 25°C), digested again with proteinase K and deproteinized as above. After ethanol precipitation, DNA was extensively dialyzed against TE.

Mouse satellite DNA was isolated by isopycnic centrifugation in Ag^+ containing Cs_2SO_4 density gradients [29].

S1 nuclease (Sigma) was used at 1 unit/μg DNA for 2 hr at 37°C in the presence of 250 mM NaCl, 30 mM Na-acetate buffer, pH 5.0, and 2 M $ZnCl_2$. After digestion with S1 nuclease, DNA was recovered by ethanol precipitation and dissolved in TE.

Digestion with EcoRI was carried out in the presence of 50 mM NaCl, 10 mM Tris·Cl, 10 mM $MgCl_2$, pH 7.5, for 1–2 hr at 37°C. Hae III was isolated according to the method of Middleton et al [30] and used in the presence of 50 mM NaCl, 6 mM Tris·Cl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg/ml BSA.

To determine the content of satellite DNA in different preparations, they were digested with BstNI (New England Biolabs, 1 unit/μg DNA, 2 hr, 45°C) in a buffer containing 20 mM KCl, 10 mM Tris·Cl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100 μg/ml BSA, pH 8.0. The reaction was stopped by addition of excess EDTA, and DNA was fractionated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and photographed in UV light. The negatives were scanned in the visible light, and the proportion of satellite DNA was determined from the area of the peaks of 234 bp monomer and higher discrete oligomers related to the total scan areas. The content of satellite DNA in total mouse DNA determined by this method was $10 \pm 2\%$.

Sucrose Gradient Centrifugation

The proportion of [^{3}H] DNA complexed in vitro with NL proteins was estimated from the radioactivity found in the pellet (percent of the input radioactivity) after

centrifugation in HSE containing linear 5–20% sucrose gradients for 1 hr at 30,000 rpm (Beckman SW 40 rotor).

Filter Binding Assay

For this assay [31], GF/B Whatman filters were mounted on a support and washed with 1.5 M NaCl in TE. Aliquots of the samples were applied and allowed to pass by gravity. The filters were then washed with 1.5 M NaCl in TE, followed by TE, dried and counted in a toluene-based scintillator. Other aliquots were counted in a scintillation cocktail containing Triton X-100 to determine the total input radioactivity. The results were corrected for the different counting efficiencies in the two scintillators and expressed as percent of input DNA, after subtracting the background retention of DNA in the absence of protein.

Gel Electrophoresis

DNA was fractionated in agarose slab gels containing 80 mM Tris·phosphate, 8 mM EDTA, pH 8.0, stained with ethidium bromide and photographed in UV light.

Protein was determined by the method of Lowry [32].

Metrizamide Density Gradients

Equilibrium centrifugation in metrizamide (MA) [33] was carried out using 7 ml 19–46% preformed gradients containing 1.5 M NaCl, 10 mM Tris·Cl, 1 mM EDTA, 0.1% BSA and 0.1 mM PMSF, pH 8.0. Samples in 50% MA, 1.5 M NaCl, 10 mM Tris·Cl, 1 mM EDTA, pH 8.0, were introduced at the bottom of the tubes and centrifuged at 39,000 rpm for 60 h in an SW 41 Beckman rotor at 4°C. The gradients were fractionated from the bottom, taking 0.3-ml fractions. The density of the fractions was determined as described elsewhere [33].

Non-equilibrium MA gradients were prepared in 11-ml SW 41 rotor tubes (Beckman) and consisted, from top to bottom, of 1 ml 55% MA overlayed with a 5 ml linear 25–45% MA gradient (densities from 1.15 to 1.26 g/cm^3) and 1.5 ml 10% sucrose. All layers contained 1.5 M NaCl, 10 mM Tris·Cl (pH 8.0), 1 mM EDTA, 0.1% BSA, and 0.1 mM PMSF. Four-milliliter chromatin samples were layered on the gradients, and the tubes were centrifuged for 3 hr at 39,000 rpm at 4°C, followed by fractionation from the bottom. These gradients combine velocity sedimentation with a density barrier to free DNA and DNA associated with small amounts of protein.

Electron microscopy was performed as described in [28] .

RESULTS AND DISCUSSION

Binding of DNA to NL In Vitro

RS preparations used in the following experiments have been characterized in [28] as representing nuclear lamina structures. Under the present conditions of digestion (Materials and Methods), NL-fraction contained 6–8% of the total nuclear DNA.

We measured the extent of binding of purified ^{3}H-labeled DNA to these structures in vitro under conditions employed in the conventional procedure for isolation of nuclear matrices [34]. The DNA was reacted with NL in two ways: (a) at NaCl concentrations below 0.1 M [low salt (LS) conditions], and (b) in solutions of 1.5–2.0 M NaCl [high-salt (HS) conditions]. In all cases, the amount of complexes was estimated in the presence of 1.5–2.0 M NaCl, and the results were corrected for the sedimentation of similarly fragmented DNA in the absence of protein. The experimental conditions and the results are given in Table I.

Under LS conditions, DNA and NL form complexes resistant to 2 M NaCl. Denatured DNA and short-pulse-labeled DNA show particularly high levels of binding. Most of the binding sites are single-stranded regions, as evidenced by the S1 nuclease results. The blunt ends produced by Hae III are not efficient binding sites. The binding is not sequence specific—ie, does not involve in vivo binding sites at least in its major part, since bacterial DNA shows complexing comparable to that of mouse total DNA or satellite DNA [26,35]. In all cases, the complex formation is strongly reduced when the reaction between DNA and NL occurs in 2 M NaCl regardless of the presence of Mg^{++} or the strandedness of DNA.

If we analyze the published studies of DNA associated with RS from a methodological point of view, it appears that in many cases [5–9,13,23] conditions have

TABLE I. In Vitro Binding of DNA to RS*

	Percent binding			
	LS conditions		HS conditions	
Exogenous [^{3}H] DNA	Sucrose gradient	Filter retention	Sucrose gradient	Filter retention
Mouse ds	41.6	22.4	1.2	0.7
E coli ds	28.0	15.4	0.8	0.7
Mouse ss	n.d.	96.2	0.3	0.5
E coli ss	84.1	97.0	0.3	0.8
Mouse ds, pulse labeled for 30 sec	n.d.	71.8	n.d.	n.d.
Mouse ds, pulse labeled for 120 sec	54.9	64.3	0.5	1.0
Mouse ds, treated with S1 nuclease	n.d.	3.6	n.d.	n.d.
Mouse ds, pulse labeled for 120 sec treated with S1 nuclease	n.d.	8.4	n.d.	n.d.
Mouse ds satellite	17.4	16.8	n.d.	n.d.

*Samples of NL containing 100 μg protein were mixed with 5 μg ^{3}H DNA in a total volume of 1 ml.
LS conditions: The mixtures were treated with Hae III (BstN1 in the case of satellite DNA) under conditions given in Methods. This resulted in a broad size distribution of DNA fragments in the range 200–4,000 bp. More than 60% of satellite DNA was in the form of 234 bp monomer. After the incubation EDTA was added to 20 mM followed by an equal volume of 4 M NaCl, 20 mM Tris·Cl, 2 mM EDTA pH 8.0. The amount of DNA complexed with NL-proteins was estimated either by sedimentation in sucrose gradients or by retention on glass fiber filters. The average size of both mouse and E coli DNA after heat denaturation (96°C, 10 min) was about 2 kb.
HS conditions: All DNA preparations used were digested with Hae III in the absence of protein, the reaction was stopped by addition of EDTA to 20 mM and DNA was mixed with NL in the presence of HSE, incubated for 2 hr at 37°C and analyzed as above; n.d. means nondetermined.

existed for in vitro formation of complexes. The increased efficiency of binding of single-stranded DNA (see also [26,35]) and that of pulse-labeled DNA may be relevant to the fact that both replicating and transcribing DNA contain regions of strand separation and both have been found to be preferentially associated with the nuclear matrix [1–3,10,12–14,20]; but results differ [11,23,36].

Chromatin Condensation Affects the Composition of DNA Bound to RS

It is to be expected that DNA located in the condensed chromatin should be less exposed to nuclease digestion than the rest of DNA and, therefore, may be found enriched in the fraction of RS. To study this problem, we took advantage of the fact that, in the mouse, satellite DNA is a specific marker of the centromeric regions of the mitotic chromosomes, which correspond to the condensed chromatin blocks at the periphery of the interphase nucleus [37–39], and we carried out the experiment outlined in Figure 1. When EAT nuclei are digested in the presence of divalent cations, which favors chromatin condensation [40], a high proportion of satellite DNA is found hydrophobically complexed with RS (samples 3 and 4). This might be interpreted to indicate that satellite DNA was preferentially attached to the nuclear matrix, as suggested by others [8,9,21,22]. According to the results with sample 1, however, this is not the case, since if digestion is carried out while chromatin is decondensed in EDTA or if chromatin is fragmented by DNAse II in EDTA prior to addition of divalent cations (sample 2), the proportion of satellite DNA in the pellet does not exceed that in total DNA. These results cannot be related to irreversible detachment of satellite DNA upon treatment of undigested nuclei with EDTA (samples 1 and 2), because after recondensation in MG^{++} (sample 3) a high proportion of satellite DNA appears in the pellet. Moreover, the final treatment with HSE does not detach the satellite DNA in samples 3 and 4.

These results demonstrate that in EAT nuclei satellite DNA is not preferentially associated with RS but may be enriched in this fraction due to protection by local chromatin condensation. In general, it is conceivable that the nonuniform accessibility of DNA to nucleases due to chromatin condensation or other reasons may result in a biased population of DNA associated with RS.

Large Aggregates in Solution of Purified DNA

We have observed that many preparations of purified DNA contain variable amounts of fast-sedimenting aggregates. Such aggregates are formed when solutions of high-molecular-weight DNA are frozen, presumably because zones of extremely high concentrations of DNA are created. Some properties of these aggregates are illustrated in Table II. They appear insoluble in 2 M NaCl, 30 mM $MgCl_2$ but are partly dissociated in TE. Fragmentation or heat denaturation of DNA disperses most of these aggregates.

It may be argued that when nuclei are dehistonized in 2 M NaCl prior to digestion [2–5,9–13,21,23,41,42] essentially undegraded DNA is liberated in a very high concentration still associated with tightly bound nonhistone proteins, perhaps in a specific manner [43]. The concentration of DNA in a nucleus [44] is 12 pg in 500 μm^3 or 24 mg/ml. Under these unique conditions (see also [45]), it is possible that formation of DNA aggregates takes place, much the same way as in solutions of purified DNA. This process may well be favored by nonhistone proteins tightly bound

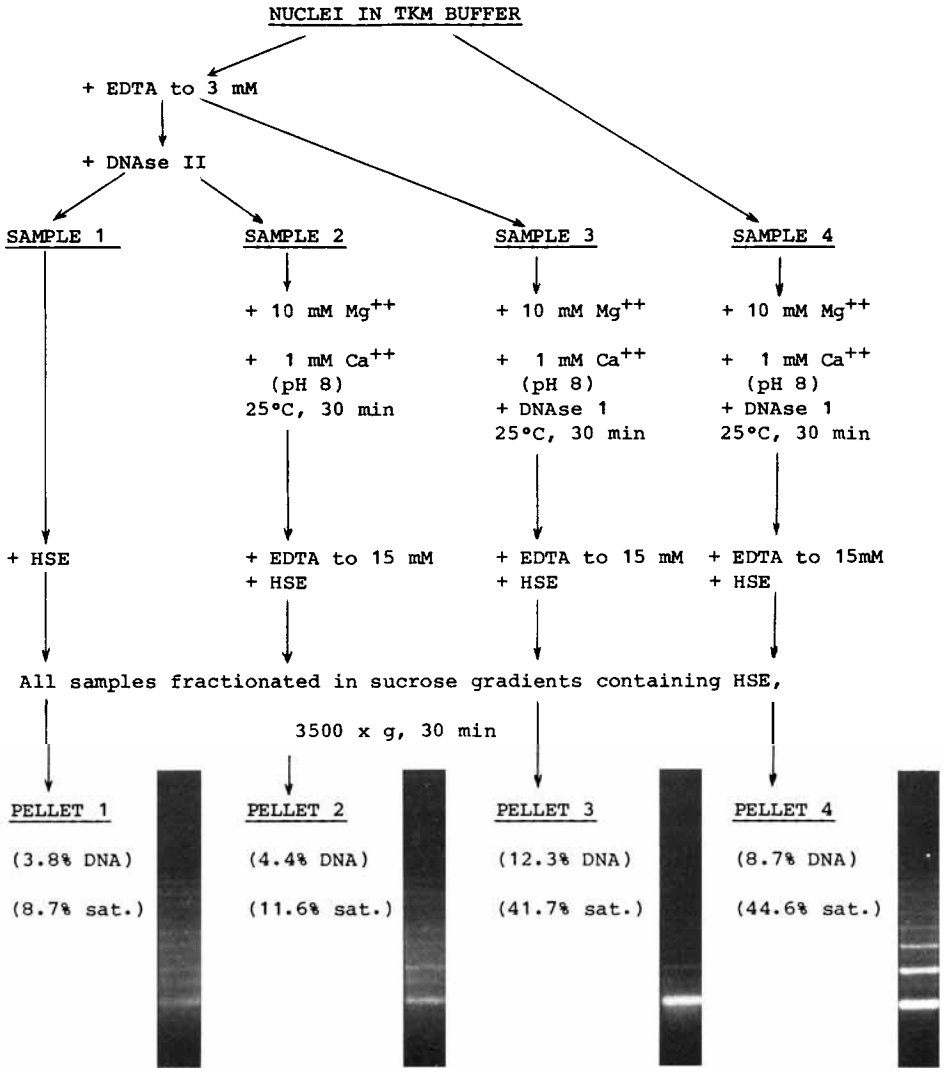

Fig. 1. Crude nuclear fraction in TKM buffer was divided into 4 aliquots. Three of them (samples 1–3) were treated with EDTA (3 mM final concentration, 30 min at 25°C) and centrifuged at 3,500g for 10 min. Two of the pellets were resuspended in TE pH 7.0 and incubated for 30 min at 25°C with DNAse II (2 units/A_{260}); pH was then adjusted to 8.0 by addition of Tris·Cl to 50 mM (samples 1 and 2). The third pellet was resuspended in 50 mM Tris·Cl, pH 8.0, supplied with divalent cations and digested with DNAse I (0.2 $\mu g/A_{260}$)(sample 3). A sample of untreated nuclei was likewise digested with DNAse I (sample 4). After sedimentation in 5–20% HSE containing sucrose gradients DNA was purified from all pellets. Its size ranged between 1 and 15 kbp in all samples. The electrophoretic profiles in 1.5% agarose gel of DNA isolated from the pellets and digested with BstNI are shown, together with the content of DNA (percentage of the input) and the proportion of satellite DNA in each pellet.

TABLE II. Effect of Different Treatments on the Fast Sedimenting Complexes in Solutions of Purified DNA*

Treatment of DNA	Incubation and sucrose gradients in:	Percent DNA in the sediment
None	2 M NaCl, 30 mM $MgCl_2$, TE	16.8
None	TE	6.2
Digestion with Hae III	2 M NaCl, 30 mM $MgCl_2$, TE	1.9
Heat denaturation	2 M NaCl, 30 mM $MgCl_2$, TE	0.3

*Purified mouse [^{3}H] DNA with average fragment size about 20 kbp was frozen at −20°C in TE at a concentration of 100 μg/ml. After thawing at room temperature, the solution was vortexed for 30 sec. Portion of the sample containing 10 μg DNA each were subjected to (a) no treatment; (b) digestion with Hae III; and (c) heat denaturation. The samples were brought to 2 M NaCl, 30 mM $MgCl_2$, TE or TE alone as indicated in a total volume of 1 ml, incubated for 30 min at 37°C, applied to 5–20% sucrose gradients and centrifuged for 30 min at 15,000 rpm in a Beckman SW40 rotor at 4°C. The gradients were fractionated, the tubes were carefully drained, and the sediments were dissolved in 1% SDS by heating and counted. Digestion with Hae III and heat denaturation reduced the average size of DNA to about 2 kbp (kb).

to specific regions of DNA [43]. Such aggregates may contaminate the pellet of RS but should be separated from it by equilibrium centrifugation.

The results described so far should allow us to define the conditions expected to minimize the artifacts inherent in the studies of DNA bound to RS. (a) The enzymatic fragmentation of DNA should be carried out under conditions of uniform accessibility of chromatin DNA and should occur prior to dehistonization. In this respect a unique possibility is offered by DNAse II, which does not require divalent cations. (b) Preparations of dehistonized RS containing DNA should not be exposed to LS conditions, especially in the presence of divalent cations. (c) Equilibrium, rather than differential centrifugation should be used to separate RS-bound from released DNA.

Chromosomal DNA Not Tightly Bound, but Rather Loosely Attached to NL

In [28] we have described a method for isolation of NL from EAT cells, which involves digestion of decondensed structured chromatin with DNAse II, followed by dehistonization in 2 M NaCl. The method appeared to satisfy some of the requirements for avoiding the artifacts described above and was applied to study the association of chromosomal DNA with NL.

When chromatin was digested with RNAse only (control samples) and dehistonized, about 40% of the total DNA sedimented in the NL fraction. It is likely that at this stage DNA had already undergone slight fragmentation as a consequence of an endogenous DNAse II contamination in these chromatin preparations [46].

Electron microscopy of whole-mount specimens of these NL pellets revealed DNA in the form of thick fibers spreading away from the central structure (Fig. 2A). These fibers were completely removed by incubation with DNAse I (Fig. 2C). Virtually all DNA in these preparations was retained on glass fibers filters. Thus, according to the accepted criteria, DNA in these samples behaved as tightly bound to NL.

To isolate DNA fragments containing the putative sites of attachment to NL, we applied the conventional approach of gradual digestion of DNA followed by recovery of the fragment sedimenting with NL at low speed. The process was monitored by

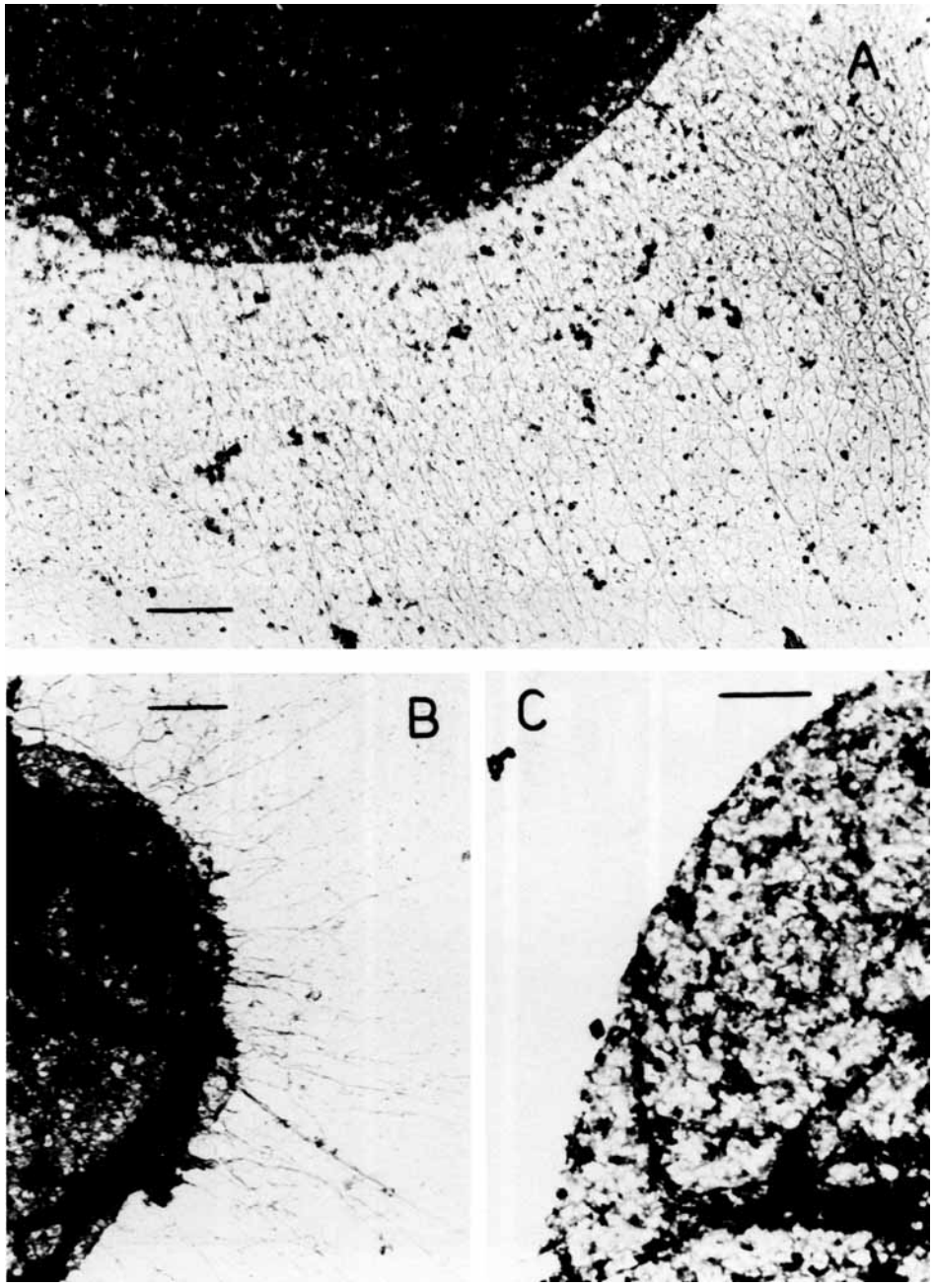

Fig. 2. Electron micrographs of whole mount preparations of NL structures isolated as described in Results. (A) after digestion of chomatin with 20 μg/ml RNAse only (control sample); (B) after digestion of chromatin with 0.6 units DNAse II per A_{260}-unit plus 20 μg/ml RNAse; and (C) the control sample shown in (A) after treatment with 10 μg/ml DNAse I in 10 mM Tris·Cl, 2 mM $MgCl_2$, pH 7.0 for 30 min at 25°C before the fixation and preparation of the specimen. Bars denote 1 μm.

measuring the amount of DNA in the pellet and its fragment size in both pellet and supernatant. At the initial stages of fragmentation both pellet and supernatant contained DNA much larger than 50 kbp (Fig. 3).

Digestion with increasing amounts of DNAse II led to a progressive release of DNA from NL. Electron micrographs of NL pellets after different extents of digestion showed lower amounts of shorter DNA fibers (Fig. 2B). At an average size of about 20 kbp, more than 99% of DNA was found in the supernatant (Fig. 3).

Further characterization of DNA cosedimenting with NL revealed that it did not differ from total DNA in its content of satellite DNA (a result similar to that in Figure 1, sample 1) or short pulse-labelled DNA (not shown) regardless of the proportion of DNA in the NL fraction.

Since a simple cosedimentation does not provide rigorous proof that DNA is tightly bound to the fast sedimenting structure (see above), DNA-NL association was studied by two independent methods, centrifugation in MA density gradients [33] and retention on glass fiber filters [31]. First, chromatin digested with DNAse II/RNAse and dissociated in 2 M NaCl was analyzed by non-equilibrium centrifugation in MA gradients (Materials and Methods). In such gradients only large structures with high

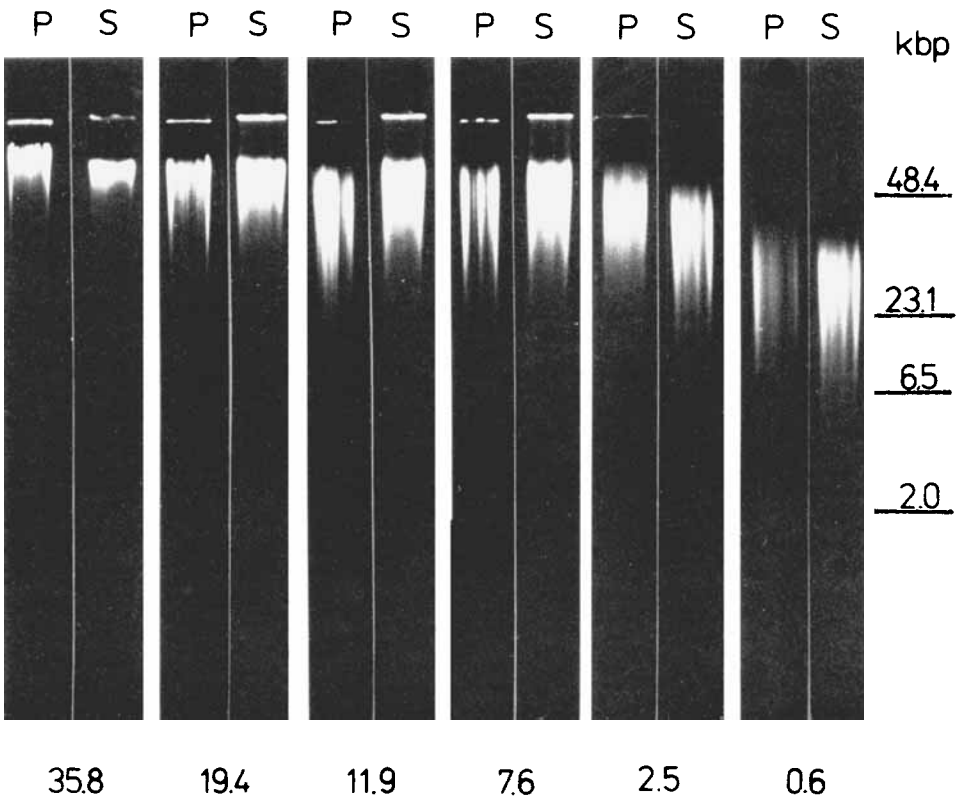

Fig. 3. The 0.4% agarose gel electrophoresis of DNA isolated from the NL fractions (P) and from the supernatant (S) after different extent of digestion of chromatin with DNAse II in the presence of 20 μg/ml RNAse. The figures below the lanes indicate the percentage of DNA in the corresponding NL fraction. Fragments of λ-phage DNA digested with Hind III were used as DNA size markers.

protein/DNA ratios would sediment to the bottom, while free DNA or deoxynucleoproteins with densities below 1.15 g/cm^3 would not be able to enter the dense MA layers. In the case of chromatin digested only with RNAse, a peak of DNA has entered the upper part of the gradient, but only 0.5% of (Fig. 4) the total protein has sedimented to the bottom, indicating that the NL is prevented from sedimenting by the large amount of DNA associated with it. Progressive digestion with DNAse II led to a shift of the DNA peak upwards and to an increase of the protein in the pellet, until at a relatively advanced fragmentation (about 20 kbp average size of DNA), the bottom fraction contained 4.2% of the protein input, a figure close to the recovery of protein in NL fraction in sucrose gradients [28]. At no stage of digestion did we observe sedimentation of DNA to the bottom of these gradients, ie association between NL and DNA stable under the conditions employed.

When centrifuged to equilibrium in 1.5 M NaCl containing MA gradients [33], DNA in pellets obtained at initial stages of fragmentation (approx. 30% of DNA in

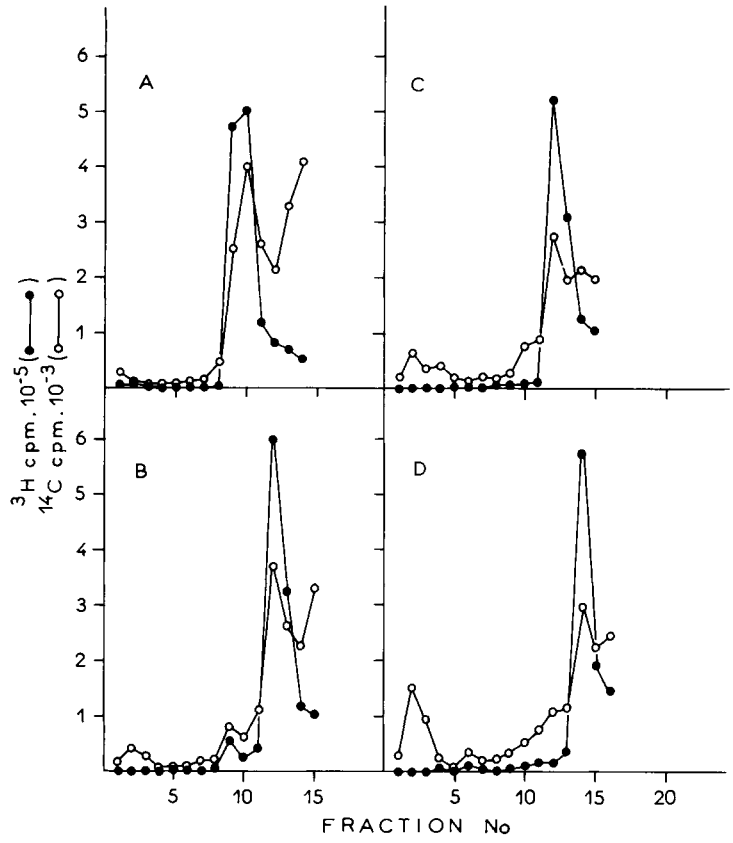

Fig. 4. Distribution of [^{3}H] DNA (●—●) and [^{14}C] protein (○—○) in nonequilibrium MA density gradients, prepared and run as described in the text. Chromatin samples were digested with 20 μg/ml RNAse plus A) no DNAse II; B) 0.5 units/A_{260} DNAse II; C) 2 units/A_{260} DNAse II; and D) 15 units/A_{260} DNAse II. The upper 4 ml of the gradients are not shown. 0.5%, 1.5%, 2.5% and 4.2% of the input [^{14}C] protein have sedimented to the bottom in the gradients shown in A, B, C, and D, respectively.

NL fraction) banded at 1.20 g/cm ^{3}, a density indicative of deoxynucleoprotein (Fig. 5A). At more advanced stages of digestion (1–4% of DNA with 20–30 kbp average size in NL fraction), we detected no DNA banding at the density of NL (approx. 1.36 g/cm^3). Instead, all DNA concentrated sharply at a density close to that of free DNA (1.14 g/cm^3) (Fig. 5B). Filter retention results confirmed that this DNA was not bound to protein to any significant extent (retention 3.7%), while 86% of DNA in the 1.20 g/cm^3 peak (Fig. 5A) was retained. The material recovered from the peak of free protein in both types of MA gradients appeared in the electron microscope as discrete NL structures and exhibited an SDS gel pattern typical for NL (see Fig. 2A and Fig. 3 in [28]), indicating that the complete release of DNA from NL was not due to desintegration of the latter in MA solutions.

Omission of EDTA from the procedure of chromatin isolation, NL isolation and subsequent analysis did not alter the results concerning DNA.

DNA-NL Association Irreversibly Stabilized by Mg^{++}

As shown above, we have been unable to detect complexes between NL and DNA resistant to 2 M NaCl, after a partial digestion with DNAse II in the absence of divalent cations. Such complexes, however, could be generated in vitro when NL-

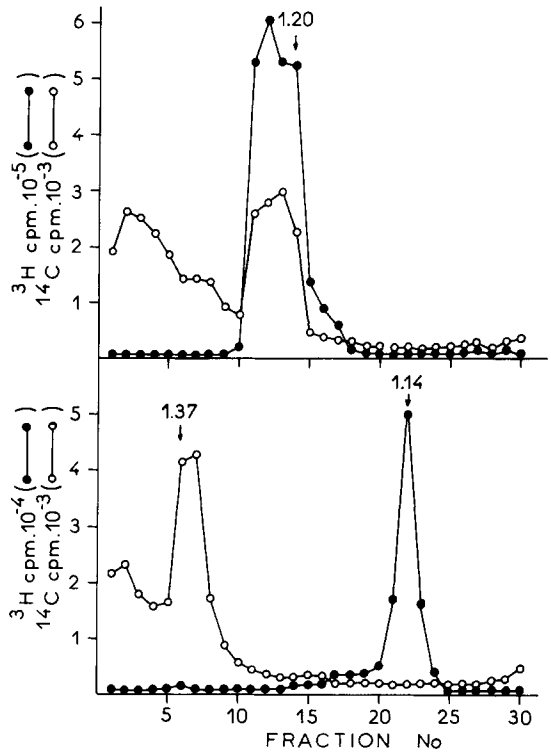

Fig. 5. Distribution of [^{3}H] DNA (●—●) and [^{14}C] protein (○—○) in equilibrium 19–46% MA gradients. The NL-fraction was obtained from chromatin samples digested (A) with RNAse only (20 μg/ml) and (B) with 10 units/A_{260} DNAse II plus 20 μg/ml RNAse. The figures above the peaks indicate the density of the fraction.

DNA complexes were digested at low ionic strength in the presence of Mg^{++}, as shown by the following experiments.

Chromatin digested with RNAse only was dissociated in 2 M NaCl and centrifuged at 39,000 rpm for 3 h through a layer of 20% sucrose containing HSE. About 40% of the input DNA sedimented. Following a brief dialysis against TE, the pellet was divided in two parts. One was digested with EcoRI (Methods) and the other with DNAse I (0.01 μg enzyme/A_{260}, 60 min, 37°C, in the presence of 10 mM Mg^{++}). After addition of EDTA to 20 mM, the samples were centrifuged again through 20% sucrose in HSE. The pellets contained about 5% of the input protein (mainly lamins A, B, and C) and about 5% of the input DNA. In the EcoRI treated sample 53% of this DNA was satellite. Similar results were obtained when Hae III was used instead of EcoRI (not shown). The pellets were further analyzed by equilibrium centrifugation in HSE containing metrizamide gradients. Under these conditions in both samples two DNA containing peaks were formed (Fig. 6). The heavy peak (1.35 g/cm^3) contained about 50% of the input DNA, more than 80% of which was retained on filters. The light peak was mostly free DNA (1.14 g/cm^3, 3.5% retention on filters).

The DNA was purified from these peaks and analyzed by agarose gel electrophoresis before and after digestion with BstNI (Fig. 7). Before the digestion, DNA from the heavy peak had an average size of about 15 kbp and that of the light peak about 1 kbp. Electrophoresis of the digested samples revealed that the heavy peak in the sample treated with EcoRI was strongly (in some experiments up to 80%) enriched in satellite DNA sequences, while the light peak contained less than 10% of such sequences. In the case of samples treated with DNAse I, both peaks contained about 10% satellite DNA (not shown).

The association of chromatin with the envelope of the interphase nucleus has been well documented [47], for review see [15,16,48,49]. In a few instances, DNA has been found attached to residual nuclear structures consisting mainly of NL [17,50] after treatment with 2 M NaCl, and it has been proposed that specific DNA sequences form direct tight hydrophobic bonds with NL proteins in vivo. This idea has been

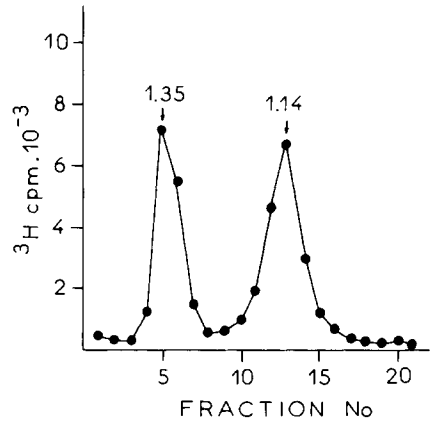

Fig. 6. Distribution of [^{3}H] DNA in equilibrium 19–46% MA gradient. The NL fraction was obtained with EcoR1 from 2 M NaCl-treated chomatin structures. The heavy peak (1.35 g/cm^3) corresponds to DNA complexed with protein and is enriched in satellite DNA (see Fig. 7, lane 3).

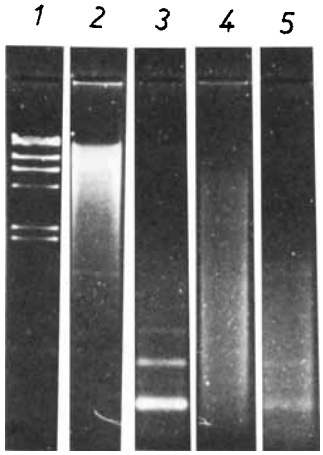

Fig. 7. The 1.5% agarose gel electrophoresis of DNA isolated from the heavy (1.35 g/cm^3) and light (1.14 g/cm^3)peaks of the MA gradient (see Fig. 6). Lanes: 1) DNA size marker, λ-phage DNA digested with Hind III; 2) DNA isolated from the 1.35 g/cm^3 peak; 3) DNA isolated from the 1.35 g/cm^3 peak and digested with BstN1; 4) DNA isolated from 1.14 g/cm^3 peak; 5) DNA isolated from the 1.14 g/cm^3 peak and digested with BstN1.

supported by the finding that the NL proteins and lamin A in particular are strong in vitro DNA binding proteins [15,27].

Our results, however, do not favor the existence of such bonds in vivo. We have been unable to isolate relatively short fragments of DNA tightly bound to NL. Instead, we find that DNA is completely released as soon as it is moderately fragmented. A separation effect, due to buoyant density differences between DNA and NL, may have contributed to the complete release of DNA, but it is unlikely that tight protein-DNA complexes, had they existed in vivo, would have dissociated in MA.

Our data, as well as most published results, are compatible with the following model (Fig. 8). According to it, chromatin DNA is not tightly bound, but rather loosely interwoven with the lamina layer. The DNA may be additionally held inside the shell in the form of a huge network by side to side interactions, possibly involving elements of the nuclear ribonucleoprotein structures, as suggested for DNA in the nucleolus [51]. When chromatin is fragmented at low ionic strength in the absence of divalent cations and then dehistonized, DNA is completely released. However, when the fragmentation occurs while the nuclear structures are condensed, relatively short DNA fragments remain tightly bound to NL or other skeletal elements as a result of secondary stabilization and artifactual in vitro binding (Table I). In agreement with this model, we have found that isolated NL shells contract to approximately 55% and 45% of the surface they have in the presence of EDTA when treated with 10 mM Mg^{++} and 10 mM Ca^{++}, respectively [Krachmarov et al, unpublished].

The tightly bound DNA fragments found in some experiments [52,53,54,55] are not necessarily a random DNA population. They may be enriched in certain sequences that have been protected by local chromatin condensation (Fig. 1) or by using enzymes like EcoRI and Hae III that leave satellite DNA intact [56,57] (Fig. 7). It has been shown that satellite DNA has a specific location in the nucleus

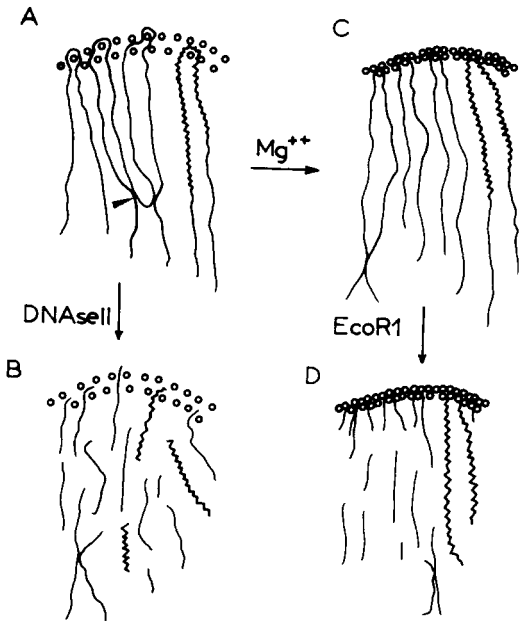

Fig. 8. Model of association of DNA with NL. A) NL and DNA in the presence of EDTA without DNAse treatment; B) NL and DNA in the presence of EDTA after partial (20–50 kbp) digestion with DNAse II (DNA is completely released); C) NL and DNA in the presence of divalent cations (condensed state) without DNAse treatment; D) NL and DNA in the presence of divalent cations after digestion with EcoR1 (some DNA remains tightly bound to NL). Open circles represent NL; zig-zag regions of DNA threads correspond to satellite DNA. The arrowhead points to possible side to side DNA interactions.

[37–39]. In this sense, the composition of DNA found tightly bound to skeletal structures may reflect certain features of the in vivo chromatin organization. According to our model, depolymerization of NL during mitosis [58] would spontaneously release the chromatin from the nuclear envelope.

Our conclusions are in agreement with the elegant experiments of Forbes et al [59], who have shown that a typical nuclear envelope, consisting of membrane, lamina, and pore complexes, is formed around phage DNA injected into *Xenopus* eggs, suggesting that no specific sequences are required for interaction between NL and chromatin. It seems probable that certain gross structural features of chromatin are recognized in this interaction in vivo, possibly involving protein factors. The 31 kdalton protein, described recently by McKeon et al [60], which undergoes a DNA size-dependent release from NL and also binds to mitotic chromosomes, may be a suitable candidate for such a role.

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